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ATTORNEY'S DOCKET NUMBER: SWA-XXX

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

U S APPLICATION NO (If known, see 37 CFR 1 5): 09/806110

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CA99/00895	27 SEPT 1999	28 SEPT 1998

TITLE OF INVENTION
USE OF PEX IN THE TREATMENT OF METABOLIC BONE DISEASES

APPLICANT(S) FOR DO/EO/US
ANDREW C. KARAPLIS, ET. AL.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☑ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☑ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☑ has been communicated by the International Bureau. (Convenience copy provided)
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☑ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☑ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☑ Other items or information: International Preliminary Examination Report accompanied by annexes. (Convenience copy provided)

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/806110	PCT/CA99/00895	SWA-XXX

21. ☑ The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	- 20 =	0	x **$18.00**	$
Independent claims	- 3 =	3	x **$80.00**	$ 240.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$
TOTAL OF ABOVE CALCULATIONS =				$ 1,100.00
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.			+	$
SUBTOTAL =				$ 950.00
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$
TOTAL NATIONAL FEE =				$ 950.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property			+	$ 40.00
TOTAL FEES ENCLOSED =				$ 990.00
Amount to be refunded:				$
charged:				$

a. ☐ A check in the amount of $ _____ to cover the above fees is enclosed.

b. ☑ Please charge my Deposit Account No. 08-0219 in the amount of $ 990.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☑ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 08-0219 . A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

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33,923
REGISTRATION NUMBER

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Andrew C. Karaplis, et.al.

Serial No.: TBA Examiner: TBA

Filed: Herewith Group Art Unit:TBA

For: Use of PEX in the treatment of Metabolic Bone Diseases

Attorney Docket No.:

Box PCT
Commissioner for Patents
Washington, D.C. 20231

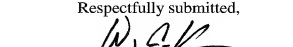
STATEMENT UNDER 37 C.F.R. §1.821(f)

Sir:

The diskette enclosed herewith contains a computer readable form of the Sequence Listing for the above-referenced patent application. The material on this diskette is identical in substance to the Sequence Listing filed herewith. The computer readable form of the Sequence Listing contained on this diskette is understood to comply with the requirements of §1.821(f).

Dated: March 28, 2001

Respectfully submitted,



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USE OF *PEX* IN THE TREATMENT OF METABOLIC BONE DISEASES

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to the use of *PEX* in the treatment of metabolic bone diseases, such as osteomalacia and osteoporosis.

(b) Description of Prior Art

Mutations in the *PEX* gene are responsible for X-linked hypophosphatemic rickets (HYP). To gain insight into the role of *PEX* in normal physiology we have cloned the human full-length cDNA and studied its tissue expression, subcellular localization, and peptidase activity. We show that the cDNA encodes a 749 amino acid protein structurally related to a family of neutral endopeptidases that include neprilysin (NEP) as prototype. By Northern blot analysis, the size of the full-length *PEX* transcript is 6.5 kb. *PEX* expression, as determined by semi-quantitative PCR, is high in bone and in tumor tissue associated with the paraneoplastic syndrome of renal phosphate wasting. *PEX* is glycosylated in the presence of canine microsomal membranes and partitions exclusively in the detergent phase from Triton X-114 extractions of transiently transfected COS cells. Immunofluorescence studies in A293 cells expressing *PEX* tagged with a c-*myc* epitope show a predominant cell-surface location for the protein with its C-terminal domain in the extracellular compartment, substantiating the assumption that *PEX*, like other members of the neutral endopeptidase family, is a type II integral membrane glycoprotein. Cell membranes from cultured COS cells transiently expressing *PEX* efficiently degrade exogenously added PTH-derived peptides, demonstrating for the first time that recombinant *PEX* can function as an endopeptidase. *PEX* peptidase activ-

ity may provide a convenient target for pharmacological intervention in states of altered phosphate homeostasis and in metabolic bone diseases.

X-linked hypophosphatemic rickets (HYP) is the most common inherited disorder of renal phosphate wasting characterized by severe hypophosphatemia, renal phosphate wasting, reduced serum concentrations of 1,25-dihydroxyvitamin D levels, and defective bone mineralization. Until recently, much of our understanding of HYP has been facilitated by the availability of two murine homologues, the *Hyp* and *Gy* mice, which exhibit many of the phenotypic features of HYP. Through positional cloning, however, a gene which spans the deleted region Xp22.1 in HYP patients, or is mutated in non-deletion patients with the disorder, was identified (designated *PEX*) and its partial cDNA sequence reported (The HYP Consortium (1995) *Nature Genetics* **11**, 130-136). The predicted human *PEX* gene product, as well as its murine homologue (Du, L. et al. (1996) *Genomics* **36**, 22-28), exhibit homology to a family of neutral endopeptidases involved in either activation or degradation of a number of peptide hormones. It has been postulated that *PEX* metabolizes a peptide hormone that modulates renal tubular phosphate handling. Such an activity could involve either the processing of a phosphate-reabsorbing hormone precursor to its active form or the inactivation of a circulating phosphaturic factor. These speculations notwithstanding, the physiologic function of the *PEX* gene product and the mechanisms that lead to the renal and skeletal abnormalities of HYP remain to be defined.

Oncogenous hypophosphatemic osteomalacia (OHO) is a rare acquired disorder of phosphate homeostasis with biochemical and physical abnormalities similar to HYP. This syndrome is associated with a variety of his-

tologically distinct, usually benign, mesenchymal tumors whose excision promptly reverses the metabolic abnormalities and results in cure of the bone disease. It is generally thought that a factor(s) produced by these tumors promotes phosphaturia and inhibits the renal conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D. The nature of the phosphaturic substance remains unknown and is likely distinct from both parathyroid hormone (PTH) and calcitonin, two polypeptide hormones known to inhibit the renal tubular reabsorption of phosphorus. Because of the striking similarity in the clinical presentation of patients with OHO and HYP, it is postulated that the factor causing phosphaturia in OHO is the active form of the *PEX* substrate. The identification and characterization of the putative *PEX* substrate, referred to as phosphatonin, however, will require first a better understanding of *PEX* function.

To date, there is still a need to understand how local factors produced in the bone regulate bone formation and bone resorption. Derangement of these factors leads to metabolic bone diseases. Pharmacological manipulation of such factors may serve as a novel approach to the treatment of these disorders.

It would be highly desirable to be provided with a tool in the treatment of metabolic bone diseases, such as osteomalacia and osteoporosis.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a tool in the treatment of metabolic bone diseases, such as osteomalacia and osteoporosis.

Another aim of the present invention is to provide the use of *PEX* in the treatment of metabolic bone diseases, such as osteomalacia and osteoporosis.

Another aim of the present invention is to provide a method of diagnostic of metabolic bone diseases, such as osteomalacia and osteoporosis.

Toward this objective, we have cloned a cDNA encoding the full-length human *PEX* protein, and determined the tissue distribution of *PEX* transcripts. In addition, we have examined the subcellular localization of recombinant *PEX* protein and demonstrated its peptidase activity.

In accordance with the present invention there is provided a method for the diagnosis of metabolic bone diseases in a patient, which comprises the step of determining the level of PTHrP in a biological sample of a patient wherein an alteration of PTHrP levels from that of a normal individual is indicative of metabolic bone diseases and/or metabolic bone diseases predisposition.

In accordance with the present invention there is provided a method for the treatment of metabolic bone diseases, which comprises administering to a patient a compound for the modulation of *PEX* enzymatic activity.

In accordance with the present invention there is provided the use of a compound for the modulation of *PEX* enzymatic activity for the manufacture of a medicament for treating metabolic bone diseases.

In accordance with the present invention there is provided a method for the treatment of metabolic bone diseases, which comprises modulating PTH and PTHrP levels that regulate osteoblast activity in a patient to modulate bone breakdown and bone formation.

In accordance with the present invention there is provided the use of modulation of PTH and PTHrP levels that regulate osteoblast activity for the treatment of metabolic bone diseases.

In accordance with the present invention there is provided a non-human transgenic mammal to study the role of PEX in bone development and homeostasis, whose germ cells and somatic cells contain a PEX gene construct for expression of PEX in osteoblast consisting essentially of a recombinant PEX gene sequence under the control of a proximal promoter of a pro-al(I) collagen gene, the PEX gene construct being introduced into the mammal, or an ancestor of the mammal, at an embryonic stage.

The non-human mammal is preferably a mouse and the proximal promoter is preferably murine pro-al(I) collagen gene, more preferably a 2.3 kb fragment thereof.

For the purpose of the present invention the following terms are defined below.

The expression "metabolic bone diseases" includes, without limitation, osteomalacia, osteoporosis, osteopetrosis and Paget's disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates *PEX* mRNA expression in OHO tumors;

Fig. 2A illustrates human *PEX* cDNA cloned from OHO tumors (SEQ ID NOS:1-2);

Fig. 2B illustrates human *PEX* and human NEP protein alignment (SEQ ID NOS:3-4);

Fig. 2C illustrates the TMpred output for PEX;

Fig. 3 illustrates *PEX* expression in human tissues;

Fig. 4 illustrates a Northern blot analysis of *PEX* mRNA;

Fig. 5 illustrates *in vitro* translation of human *PEX* cRNA;

Figs. 6A-6B illustrate TRITON™ X-114 extraction and immunofluorescent localization of *PEX*;

Figs. 7A-7C illustrate HPLC analysis of the hydrolysis of [D-Ala^2,Leu^5]enkephalin;

Figs. 8A-8C illustrate the hydrolysis of PTH-derived peptides by *PEX* endopeptidase activity; and

Fig. 9 illustrates Schematic representation of phosphate handling in the proximal renal tubule in normal, OHO, and HYP states.

DETAILED DESCRIPTION OF THE INVENTION

PEX is a Cell Membrane-Associated Protein

Previous studies have established that NEP, ECE-1 and Kell blood group glycoprotein are integral membrane proteins. We have used extraction with the detergent TRITON™ X-114 and immunochemical localization to examine whether *PEX* is also a membrane-associated protein. For identification of *PEX*, we generated a construct in which the carboxyl terminus sequences of *PEX* are modified by a human c-myc tag. The epitope tag was inserted immediately upstream of the potential prenylation motif so that any lipid modification of the *PEX* protein may proceed uninterrupted.

TRITON™ X-114 is a detergent that forms an aqueous solution at 4°C but separates into hydrophobic and aqueous phases when the temperature is raised to 30-37°C. This property has been used as an indicator of the hydrophobic nature of proteins, with integral membrane proteins partitioning exclusively in the detergent phase while highly hydrophilic proteins associate with the aqueous phase. TRITON™ X-114 extracts from COS-7 cells transiently expressing *PEX* tagged with the c-myc epitope showed that *PEX* partitions nearly exclusively into the detergent phase. This finding indicates that *PEX* is a

membrane-associated protein and is consistent with the prediction from sequence analysis that it is an integral membrane protein.

To determine the subcellular localization of *PEX*, the distribution of recombinant protein expressed in stably transfected A293 cells was examined using immunofluorescence. When cells were fixed and permeabilized, myc-tagged *PEX* immunostaining was detected primarily on the cell surface, but in a number of cells staining was also observed intracellularly, although no signal was observed in the nucleus. If permeabilization was omitted, staining was localized exclusively to the plasma membrane, while untransfected cells or cells transfected with vector alone showed no immunofluorescent staining. Since the myc-tag was inserted in the carboxyl end of *PEX*, these findings further corroborate the sequence-based prediction that *PEX* is a Type II integral membrane protein with its large C-terminal hydrophilic domain containing the active enzymatic site in the extracellular compartment.

Recombinant PEX protein has peptidase activity

The subcellular localization and sequence similarity between *PEX* and NEP strongly suggest that *PEX* functions as a membrane-bound metallopeptidase. However, no peptidase activity has yet been ascribed to *PEX*. As shown, when [D-Ala^2, Leu^5] enkephalin, used to assay for NEP activity, was incubated with cell membrane preparations from vector-transfected COS cells or COS cells expressing equivalent amounts of recombinant human NEP or *PEX* proteins, as determined by Western blot analysis, production of Tyr-D-Ala-Gly from the substrate was evident only in NEP-expressing membrane preparations. While the *PEX* sequence preserves two of the residues critical for catalytic activity of NEP (equivalent to E^{646} and H^{711}), it lacks a residue equivalent to R^{102} shown to be crucial for the dipeptidylcarboxypeptidase activity of NEP. Therefore,

unlike NEP, *PEX* has no dipeptidylcarboxypeptidase activity.

To test for peptidase activity of recombinant *PEX*, cell membrane preparations from vector-transfected COS cells or COS cells expressing recombinant *PEX* protein were incubated with human parathyroid hormone PTH (1-34) and PTH (1-38). As shown, *PEX* activity was able to degrade both peptides in a very characteristic pattern. Therefore, *PEX* functions as an endopeptidase, and more specifically we have shown for the first time that it degrades PTH. PTH is the first and only known substrate of *PEX*.

These observations make two important points:

PEX is a membrane bound protein with its active enzymatic site in the extracellular compartment. The cells with the highest level of *PEX* expression are the osteoblasts (bone forming cells). These cells are also the site of action of circulating PTH at the level of the bone. PTH stimulates these cells to produce factors (nature unknown) which in turn stimulate other bone cells, specifically the osteoclasts, to break down bone. Since *PEX* likely inactivates PTH in contact with osteoblasts, it would result in decreased stimulation of osteoclasts and therefore less bone breakdown.

Alternatively, osteoblasts produce parathyroid hormone-related peptide, PTHrP, which is important in the development of normal bone density. PTHrP shares many of the structural features of PTH and may therefore also serve as substrate for *PEX*. Our previous studies using PTHrP heterozygous-null mice generated by gene targeting have shown that decreased levels of PTHrP in the skeletal microenvironment lead to a premature form of osteoporosis. *PEX* in osteoblasts may therefore modulate local PTHrP levels and thus bone formation. Inhibition of *PEX* enzymatic activity may allow higher local concentrations of PTHrP and therefore better bone formation.

By examining PTH breakdown fragments, we can now design peptide and non-peptide activators and inhibitors of *PEX* enzymatic activity.

By modulating PTH and PTHrP levels that regulate osteoblast activity, *PEX* may play a critical role in the pathogenesis of osteomalacia and osteoporosis. By pharmacological modulation of *PEX* activity, it will be possible to modulate bone breakdown and bone formation. This would be a totally novel approach to the treatment of these metabolic bone diseases.

Experimental procedure

Tumor Tissues

Patient I was a 55 year-old woman who presented with a two-year history of progressively increasing bone pain and difficulty in walking. X-rays of the lumbosacral spine showed diffuse osteopenia. Biochemical investigation showed the serum calcium level to be normal while serum phosphorus was low (0.41 to 0.57 mmol/L; normal, 0.8-1.6 mmol/L). Alkaline phosphatase was 232 U/L (normal, 30-105 U/L) and tubular reabsorption of phosphate while the patient was hypophosphatemic was decreased to 63% (normal, >80%). A search for a tumor was negative and the patient was treated with 1,25-dihydroxyvitaminD3 and oral phosphate. Five years later a right hand mass was discovered and was surgically removed. On histopathological examination, it was a fibrous hemangioma. Postoperatively, the patient noted increasing strength in her lower extremities and marked decrease in her pain. The serum phosphorus normalized (0.96 mmol/L) and the tubular reabsorption of phosphate improved but did not completely normalize (71-76%). No recurrence of the tumor has been found ten years later.

Patient II was a 21 year old man with classic features of OHO. Resection of a benign extraskeletal

chondroma from the plantar surface of the foot resulted in complete reversal of the biochemical and clinical abnormalities associated with the syndrome.

Tumor tissue obtained from these two patients at surgery was frozen immediately in liquid nitrogen and stored at -70°C.

PEX Expression in OHO-Associated Tumors

RNA was extracted from tumor tissue using the RNeasy™ Total RNA kit (Qiagen, Chatsworth, CA) and reverse transcribed using oligo(dT) primer and Superscript II (BRL) reverse transcriptase for 1 hour at 42°C in a final reaction volume of 30 µl. The resulting cDNA was then amplified using human *PEX*-specific oligonucleotide primers *PEX*-1 (5'-GGAGGAATTGGTTGAGGGCG -3' SEQ ID NO:5) and *PEX*-2 (5'-GTAGACCACCAAGGATCCAG -3' SEQ ID NO:6), designed from the published cDNA sequence (1298 and 1807 are the nucleotide positions of the 5' end of the sense and antisense primers, respectively) (The HYP Consortium (1995) *Nature Genetics* **11**, 130-136). Following amplification (35 cycles), an aliquot of the PCR reaction was fractionated on an 1% agarose gel and visualized following staining with ethidium bromide.

Cloning of Full-Length PEX cDNA

Cloning of the 5' end of *PEX* cDNA was accomplished by anchored PCR. Total cellular RNA was extracted from tumor II and mRNA was prepared. 1.5 µg of mRNA was reverse transcribed into cDNA using 100 ng of a *PEX*-specific antisense oligomer (*PEX*-2) and 200 units of Superscript II (BRL) reverse transcriptase for 1 hour at 42°C in a final reaction volume of 30 µl. The resulting cDNA was size fractionated on a 1% agarose gel and fragments corresponding to >600 bp were purified and resuspended in H_2O. The 3' end of the first strand cDNA was homopolymer tailed with dGTP using 1 µl

of Terminal deoxynucleotidyl transferase (TdT) at 37°C for 30 minutes in a volume of 50 µl. Following heat inactivation of the enzyme, the RNA template was removed by incubation with RNase H and the tailed cDNA was purified by phenol-chloroform extraction followed by ammonium acetate precipitation. The purified tailed cDNA was resuspended in H_2O and an aliquot was used for anchored PCR analysis along with 200 ng of an internal *PEX* specific antisense primer (*PEX*-3, 5'-CGTGCCCAGAACTAGGGTGCCACC-3' (SEQ ID NO:7); nucleotide 98 of the published human cDNA sequence is the 5' end of the primer) and 200 ng of oligodC as the sense primer. Forty cycles of PCR were performed using 0.5 µl of Taq polymerase (Promega Biotec, Madison, WI) in a reaction volume of 50 µl. Cycling parameters were: 1 minute of denaturation at 94°C, 2 minutes of annealing at 55°C and 2 minutes of extension at 72°C. The PCR products were fractionated on a 1% agarose gel and a band of 700 bp was isolated, purified, and ligated into pPCRII vector (Invitrogen). Following transformation into INVαF' bacteria, clones containing the appropriate size insert were sequenced.

To clone the 3' end of *PEX* cDNA, an aliquot of an amplified unidirectional cDNA library in pCDNA3 vector (Invitrogen) generated from mRNA obtained from tumor I was grown overnight in LB medium and plasmid DNA extracted. DNA (0.5 µg) was subjected to PCR using a *PEX*-specific sense oligomer (*PEX*-1) and an antisense oligomer corresponding to the SP6 RNA polymerase binding site sequences present in the pCDNA3 vector. Thirty-five cycles of amplification were performed in a 50 µl reaction volume with each cycle consisting of 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C. Amplified products were fractionated on a 1% agarose gel and a 1.2 kb fragment cor-

responding to the 3' end of *PEX* cDNA was subcloned and sequenced.

For expression studies, an EcoRV (in the polylinker of pPCRII) /AccI (in the *PEX* sequence) fragment containing the 5' end of *PEX* cDNA was ligated into the pPCRII vector containing the 3' end of *PEX* cDNA following digestion with AccI and EcoRV. The resulting plasmid was restricted with KpnI and NotI excising the full length *PEX* cDNA that was then inserted into pCDNA3 vector digested at the KpnI/NotI sites in the polylinker region, resulting in plasmid p*PEX*. The full-length *PEX* cDNA was sequenced using an Applied Biosystems 373A automated sequencer.

Tissue Expression of PEX mRNA

PEX expression was examined in normal human tissues and in the Saos-2 human osteoblastic osteosarcoma cell line, by RT-PCR using oligonucleotides *PEX*-4 (5'-CTGGAT-CCTTGGTGGTCTAC-3' SEQ ID NO:8) and *PEX*-5 (5'-CACTGTGCAACTGTCTCAG-3' SEQ ID NO:9) as sense and antisense primers (2398 and 2895 are the nucleotide positions of the 5' end of these primers designed from the full-length human *PEX* cDNA). Semiquantitative PCR analysis for *PEX* expression in human tissues was performed as previously described, following normalization for *GAPDH* message in all samples containing *PEX* transcripts.

Northern-blot Analysis

Total RNA was obtained from Tumor I and human Saos-2 osteosarcoma cells using the RNeasy Total RNA kit (Qiagen) and oligo(dT)-purified poly(A)$^+$ RNA was isolated from Saos-2 total RNA using standard procedures. Twenty micrograms of Tumor I total RNA and 20 µg of Saos-2 poly(A)$^+$ RNA were fractionated on 1% denaturing agarose gel, and transferred to nylon membrane (Hybond N$^+$, Amersham). Hybridization was performed with

^{32}P-labeled full-length human *PEX* cDNA (3.1 kb) in 7 mM Tris-HCl, 50% formamide, 10% dextran sulfate, 4 X SSC, 2 x Denhardt's solution and heat-denatured salmon sperm DNA (100 µg/ml). The blot was washed in 0.1 X SSC, 0.1% SDS for 20 min at 50°C, and subjected to autoradiography for 4 days.

In Vitro Transcription, Translation, and Analysis of Products

Plasmid p*PEX* was linearized with NotI and sense RNA strand was transcribed using T7 RNA polymerase. Translation reactions in rabbit reticulocyte lysate were performed in the presence of [^{3}H]leucine according to the manufacturer's recommendations (Promega) with or without canine pancreas microsomal membranes. Products were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 8%). Autoradiography was performed after treating the gel with EN^{3}HANCE (Dupont NEN), as previously described.

Generation of myc-tagged PEX, Transfection in COS-7 Cells, and Triton X-114 Extraction

Plasmid p*PEX*-myc was generated by PCR amplification of *PEX* cDNA using oligonucleotide *PEX*Myc1 as the sense primer (5'-TTGGATGTCAACGCCTCG -3' SEQ ID NO:10, 519 is the nucleotide position of the 5' end of this primer designed from the cloned human *PEX* cDNA) and *PEX*Myc2 as the antisense (5'-CTACCACAATCTACAGTTGTT-CAGGTCCTCTTCGCTAATCAGCTTTTGTTCCATAGAGTCCATGCCTCTG-3' SEQ ID NO:11) primer. The latter encodes the human c-*myc* tag sequences (underlined) and *PEX* sequences corresponding to the carboxyl terminal of the mature protein (^{742}RGMDSMEQKLISEEDLNNCRLW*). Following PCR, the amplified fragment was ligated to the pPCR II vector, excised by digestion with KpnI/NotI and inserted into the corresponding sites in the polylinker region of pCDNA3. The in-frame fusion protein was verified by DNA sequencing.

COS-7 cells maintained in Dulbecco's modified Eagle's medium (DMEM, 4,500 mg/L glucose with L-glutamine; JRH Biosciences, Lenexa, KS) supplemented with 10% fetal calf serum (FCS; GIBCO) and antibiotics (pen/strep) were plated at a density of 3 x 10^5 cells/well in 6-well cluster plates 24 h prior to transfection. Cells were washed with twice with PBS and incubated with 2 µg of p*PEX*-myc plasmid DNA in 1 ml of DMEM containing 0.1% BSA, and DEAE-dextran (Pharmacia LKB) for 3.5 h at 37°C. Following incubation, the transfection medium was aspirated, the cells were shocked with 10% DMSO in PBS for 2 min, and then cultured in DMEM with 10% calf serum at 37°C for 48 h. Triton X-114 extraction were performed on cultured cells expressing myc-tagged *PEX* as described. The samples were then analyzed by immunoblotting using the 9E10 anti-myc monoclonal antibody.

Stable Transfection of A293 Cells and Immunofluorescence

A293 cells maintained in DMEM with 10% FCS were transfected with the p*PEX*-myc plasmid by electroporation and selection initiated using G418 (600 mg/ml for 14 days and then decreased to 400 mg/ml). Populations of stably transfected cells were recovered at the end of the selection period. For myc-tagged *PEX* indirect immunofluorescence, stably transfected cells plated on gelatin-coated coverslips were washed twice with PBS, fixed in 4% parafolmaldehyde and in some experiments permeabilized with 0.5% Triton X-100. Cells were blocked with 10% FCS in DMEM for 30 min, washed and incubated for 1 hr at 37°C with the 9E10 anti-myc monoclonal antibody (1:500 dilution). Cells were subsequently washed and incubated in turn with fluorescein-conjugated sheep anti-mouse secondary antibody (1:250 dilution). Coverslips were rinsed extensively with PBS, mounted in medium (glycerol:Tris; 1:1) containing 2.5%

1,4-diazabicyclo-(2,2,2) octane (Sigma) and examined with fluorescent microscopy using appropriate filters.

Assay for membrane-bound endopeptidase activity

COS-7 cells transiently transfected with pCDNA3 vector alone, with vector containing human *NEP* cDNA (generous gift of P. Crine, Université de Montréal), or with p*PEX* plasmid, were washed and scraped in PBS. Following brief centrifugation, the cell pellets were resuspended in 50 mM Tris-HCl, pH 7.4 and disrupted by sonication. Homogenates were fractionated by sequential centrifugation at 1,000 x g for 10 min and then at 100,000 x g for 60 min. The final precipitate was washed with 50 mM Tris-HCl, pH 7.4, resuspended in the same buffer, and assayed for endopeptidase activity. The protein concentration in membrane fractions was determined by the method of Bradford with bovine serum albumin as standard.

[D-Ala^2,Leu^5] enkephalin (500 µM) was incubated with COS cell membrane preparations (~60 µg of protein) in 100 mM Tris-HCl, pH 7.0, at 37°C for 30 min (final volume 30 µl). The reaction was terminated by the addition of 100 µl 0.1% TFA (v/v). Production of Tyr-D-Ala-Gly was monitored using reversed-phase HPLC (Bondpak C-18 reverse phase column, Waters) with a U.V. detector set at 214 nm. A linear solvent gradient of 0% B to 40% B in 60 min was used with a flow rate of 1.5 ml/min (mobile phase A=0.1% TFA (v/v); mobile phase B=80% acetonitrile/0.1% TFA). Tyr-D-Ala-Gly was identified by co-chromatography with marker synthetic peptide. For assessing *PEX* endopeptidase activity, 10 µg of PTH [1-38] and PTH [1-34] peptides (Peninsula Laboratories; Belmont, CA) were added to the membrane preparations. For HPLC analysis of hydrolysis products, a linear solvent gradient of 0% to 50% solution B was used at a

rate of 1.5 ml/min. MALDI-TOF mass spectrometry was performed on specific peptide fragments.

RESULTS

Cloning of Human PEX cDNA

At the initiation of these studies, *PEX* expression had been reported in minute amounts only in leukocytes and fetal brain. We postulated that in states of hypophosphatemia *PEX* expression may be increased and therefore opted to use the OHO tumor as a tissue source that may express considerably more *PEX*. Tissues obtained from two tumors associated with OHO were used to obtain total RNA and analysis for *PEX* mRNA expression was assessed by RT-PCR. As shown in Fig.1, *PEX* transcripts were readily amplified from both tumor samples demonstrating the expected 509 bp fragment predicted from the published partial human *PEX* sequence (The HYP Consortium (1995) *Nature Genetics* **11**, 130-136). Total RNA extracted from two tumors associated with OHO was reverse transcribed and amplified by PCR (35 cycles) using human *PEX*-specific primers, *PEX*-1 and *PEX*-2, designed from the published human sequence. The expected 509 bp amplified fragment was obtained from both tumor samples. Control, no cDNA added to the amplification reaction, i.e. negative control; Marker, Φ174 DNA digested with HaeIII restriction endonuclease.

The cloning of the 3' end of *PEX* transcript was performed by rapid amplification of the 3' end of the cDNA (3' RACE), while the 5' of the cDNA was amplified by anchored PCR, as described in Experimental Procedures. Fig. 2A shows the nucleotide and predicted amino acid sequence of the full-length human *PEX* cDNA cloned from tumor tissues. Nucleotide and deduced amino acid sequence of tumor-derived human *PEX* cDNA (Fig. 2A). The numbering begins at the 5' end nucleotide as determined by anchored PCR. Amino acids are given below each codon

using the single letter code. The putative start codon is indicated as /1 along with the deduced amino acid translation. Two stop codons preceding the predicted initiation ATG are in bold type. Asterisk (*) indicates an in-frame stop codon, while a large asterisk (*) denotes the putative prenylation site. A potential polyadenylation signal in the 3' untranslated region is underlined. Nine potential N-glycosylation sites are boxed. The sequence has been assigned GenBank accession No. (U82970).

The composite cDNA reveals a single open reading frame encoding a protein of 749 amino acids which displays homology (34.2% identity, 70% similarity) to human neprilysin (NEP; EC 3.4.24.11), and other members of the membrane-bound metalloendopeptidase family encompassing endothelin-converting enzyme-1 (ECE-1; 66% similarity) and the Kell antigen (60% similarity), suggesting that *PEX* is a novel member of this family of neutral endopeptidases, as previously suggested (The HYP Consortium (1995) *Nature Genetics* **11**, 130-136). Like the other members, *PEX* is a likely a glycoprotein with eight potential N-glycosylation sites and 10 cysteine residues that may be important for the proper folding and hence native conformation of the protein.

The ATG codon at position 604 was assigned as the initiator methionine since it is preceded by two in-frame TGA termination codons 36 and 63 basepairs upstream and conforms favorably to the Kozak consensus for vertebrate initiation of translation. The cloned cDNA identifies the first 3 and the last 108 amino acids of the predicted *PEX* gene product in addition to the published partial sequence. These additional amino acids comprise residues such as E^{642} and H^{710} that are shared by NEP, and may be critical for the formation of the active site of the protein and hence its enzymatic

activity. Three amino acid residues predicted from our cDNA clone differ from the published partial human *PEX* sequence, D363A (GAC to GCC), R403W (AGG to TGG), and A641G (GCG to GGA). To confirm that these alterations did not arise because of PCR errors, *PEX* sequences were amplified from Saos-2 human osteosarcoma cells (see below) and sequenced. In addition, the same alterations were subsequently described in the murine *PEX* cDNA, suggesting possible cloning artifacts in the published partial human *PEX* sequence. Our cloned sequences also encompass 603 nucleotides of the 5' untranslated region, and 276 nucleotides of the 3' untranslated region, including the canonical polyadenylation signal AATAAA, 19 nt upstream of the poly(A) tract. The human and the published mouse *PEX* cDNA sequences share extensive homology within the protein coding region (96% identity) as well as in the 5' and 3' non coding regions.

TMpred analysis of the human *PEX* sequence predicts that the protein has no apparent N-terminal signal sequence but has a single membrane-spanning helical domain comprising amino acid residues 21-39 (Fig. 2C). TMpred analysis of the *PEX* sequence showing a single membrane-spanning domain encompassing amino acid residues 21-39 (arrowhead). Numbers on the horizontal axis refer to the amino acid sequence. Amino acid homology between *PEX* and human NEP cDNA (Fig. 2B). Sequence comparison was performed using the LALIGN program.

This predicts its transmembrane topology to be that of a type II integral membrane protein with a 20-residue N-terminal cytoplasmic tail and a C-terminal of 700 amino acid residues containing the catalytic domain in the extracellular compartment. Unexpectantly, a CXXX box motif comprising amino acid residues ^{746}CRLW was also identified at the carboxyl terminus of *PEX*. This

motif may serve as a site for prenylation, a post-translational lipid modification involved in a number of processes including facilitating membrane attachment, targeting of proteins to specific subcellular membrane compartments, promoting protein-protein interactions and regulating protein function.

Tissue Expression of PEX mRNA

We next examined *PEX* expression in a number of fetal and adult tissues and compared the level of expression to OHO tumor RNA using semi-quantitative RT-PCR (Fig. 3). Quantitative RT-PCR amplification of the *PEX* transcripts from total RNA prepared from human tissues and OHO-associated tumor. Relative expression levels for the *PEX* transcript were measured by quantifying *PEX* product in reversed-transcribed RNA samples that have been previously normalized for GAPDH levels. The specific primers used were as follows: for *PEX*, the forward primer was *PEX*-4 and the reverse primer *PEX*-5; for GAPDH, the primers were as previously described. PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Control, negative control; Marker, Φ174 DNA digested with HaeIII restriction endonuclease. Below, shown are the relative levels of *PEX* transcripts in various human tissues compared to those in the tumor.

PEX transcripts were expressed in human fetal calvarium and to a lesser degree in fetal kidney and skeletal muscle while no expression was apparent in fetal liver. *PEX* expression was also observed in the human osteoblastic osteosarcoma cell line, Saos-2. In adult tissues, *PEX* mRNA was identified in kidney, but not in liver, or endomyocardium. Recent studies have also reported *PEX* expression in human fetal bone, skeletal muscle, and liver as well as fetal and adult ovary and lung (Beck, L. et al. (1997) *J. Clin. Invest.*

99, 1200-1209; Grieff, M. et al. (1997) *Biochem. Biophys. Res. Commun.* **231**, 635-639). Analysis following normalization for *GAPDH* message in all tissues containing *PEX* transcript disclosed that bone *PEX* expression is 2-10 fold higher than in other normal tissues examined. In comparison, OHO tumor *PEX* expression was twice the levels observed in fetal calvarium, consistent with its relative "overabundance" in these tissues.

Northern Blot Analysis

To determine the size of the full-length *PEX* transcript, we isolated total RNA from tumor I (quantity of available tissue was insufficient for poly(A)$^+$ RNA extraction) and poly(A)$^+$ RNA from human Saos-2 osteosarcoma cells. This cell line was used since it is readily available and successful amplification of *PEX* sequences has been performed by RT-PCR (see above). Aliquots (20 µg of each) were examined by Northern-blot analysis using the cloned human *PEX* cDNA as probe. A single transcript of approximately 6.5 kb was readily detected only in the Saos-2-derived poly(A)$^+$ sample and contrasts with the predicted size of the cloned sequence of 3.1 kb (Fig. 4). Approximately 20 µg of poly(A$^+$)RNA prepared from Saos-2 cells and 20 µg of total RNA prepared from tumor I tissue were resolved on 1% agarose gel containing formaldehyde and then transferred to a nylon membrane. Following hybridization with radiolabeled *PEX* cDNA, the blot was washed and the signal detected by autoradiography. A transcript of ~6.5 kb was observed only in the lane containing Saos-2 poly(A$^+$)RNA. There is suggestion of an additional band corresponding to a transcript of ~3.8 kb. Arrows indicate the position of the 28S (approx. 4.8 kb) and 18S (approx. 1.8 kb) ribosomal RNA.

This finding would therefore predict a ~4 kb 5' untranslated region for *PEX* cDNA, consistent with pub-

lished data from Northern blot analysis of *PEX* expression in mouse calvaria (Du, L. et al. (1996) *Genomics* **36**, 22-28). A less well defined band was also detected in the Saos-2 sample corresponding to a potential transcript of ~3.8 kb, although the nature of this transcript remains unclear. Northern analysis of total RNA samples from tumor I and Saos-2 cells (results not shown) did not reveal any signal for *PEX*, consistent with the relatively low expression levels of the *PEX* transcript, previously described (The HYP Consortium (1995) *Nature Genetics* **11**, 130-136; Beck, L. et al. (1997) *J. Clin. Invest.* **99**, 1200-1209; Grieff, M. et al. (1997) *Biochem. Biophys. Res. Commun.* **231**, 635-639). This finding contrasts sharply with *PEX* expression levels demonstrated in murine calvaria and cultured osteoblasts (Du, L. et al. (1996) *Genomics* **36**, 22-28) and may reflect tissue and species differences.

In vitro translation of PEX cRNA

In vitro translation studies using full-length human *PEX* cRNA were performed in the rabbit reticulocyte lysate cell-free system. In the absence of microsomal membranes, *PEX* cRNA was translated into an ~86 kD protein, as predicted from the cloned cDNA sequence (Fig. 5). Plasmid p*PEX* was linearized and sense RNA strand transcribed using T7 RNA polymerase. Translation of *PEX* cRNA was performed using rabbit reticulocyte lysate in the absence (minus) and presence (plus) of canine pancreas rough microsomes. Products were electrophoresed in a SDS-polyacrylamide gel (10%) and visualized by autoradiography. Arrowhead in lane 2 indicates full-length human *PEX* protein. The addition of microsomal membranes results in the appearance of higher molecular weight forms that likely represent glycosylated products.

Following addition of canine microsomal membranes to the translation mixture, products of higher molecular weight (~100 kD) became apparent, consistent with N-glycosylation of *PEX* at the eight potential glycosylation sites deduced from the predicted sequence.

PEX is a Cell Membrane-Associated Protein

Previous studies have established that NEP, ECE-1 and Kell blood group glycoprotein are integral membrane proteins. We have used extraction with the detergent Triton X-114 and immunofluorescent localization to examine whether *PEX* is also a membrane-associated protein. For identification of *PEX*, we generated a construct in which the carboxyl terminus sequences of *PEX* are modified by a human c-*myc* tag. The epitope tag was inserted immediately upstream of the putative prenylation motif so that any potential lipid modification of the *PEX* protein may proceed uninterrupted.

Triton X-114 is a detergent that forms an aqueous solution at 4°C but separates into hydrophobic and aqueous phases when the temperature is raised to 30-37°C. This property has been used as an indicator of the hydrophobic nature of proteins, with integral membrane proteins partitioning exclusively in the detergent phase while highly hydrophilic proteins associate with the aqueous phase. Triton X-114 extracts from COS-7 cells transiently expressing *PEX* tagged with the c-*myc* epitope showed that *PEX* partitions nearly exclusively into the detergent phase (Fig. 6A). Extraction and partitioning of *PEX* expressed in COS-7 cells with Triton X-114 (Fig. 6A). Plasmid p*PEX*-myc was transiently transfected in COS-7 cells and 48 h later cells were extracted with Triton X-114. Whole cell extracts, as well as detergent and aqueous phases, were analyzed by SDS-PAGE and immunoblotted with an anti-myc monoclonal antibody. Right margin indicates $M_r \times 10^{-3}$.

This finding indicates that *PEX* is a membrane-associated protein and is consistent with the prediction from sequence analysis that it is an integral membrane protein.

To determine the subcellular localization of *PEX*, the distribution of recombinant protein expressed in stably transfected A293 cells was examined using immunofluorescence. When cells were fixed and permeabilized, *myc*-tagged *PEX* immunostaining was detected primarily on the cell surface, but in a number of cells staining was also observed intracellularly, although no signal was observed in the nucleus (Fig. 6B). If permeabilization was omitted, staining was localized exclusively to the plasma membrane (Fig. 6C), while untransfected cells or cells transfected with vector alone showed no immunofluorescent staining. Localization of *PEX* using indirect immunofluorescence in stably transfected A293 cells with (Fig. 6B) and without (Fig. 6C) permeabilization with Triton X-100, respectively. Staining was carried out using the 9E10 anti-myc monoclonal antibody, followed by fluorescein-labeled secondary (sheep anti-mouse) antibody. Arrowheads indicate intracellular (B) and plasma membrane staining (C).

Since the *myc*-tag was inserted in the carboxyl end of *PEX*, these findings further corroborate the sequence-based prediction that *PEX* is a type II integral membrane protein with its large C-terminal hydrophilic domain in the extracellular compartment.

Recombinant PEX protein has endopeptidase activity

The subcellular localization and sequence similarity between *PEX* and NEP strongly suggest that *PEX* functions as a membrane-bound metallopeptidase. However, no peptidase activity has been ascribed to *PEX*. As shown in Fig. 7A, when [D-Ala^2, Leu^5] enkephalin, used to assay for NEP activity, was incubated with cell

membrane preparations from vector-transfected COS cells or COS cells expressing equivalent amounts of recombinant human NEP or *PEX* proteins, as determined by Western blot analysis, production of Tyr-D-Ala-Gly from the substrate was evident only in NEP-expressing membrane preparations. Cell membrane preparations from vector transfected COS-7 cells (Fig. 7A) or from cells transiently expressing human *NEP* (Fig. 7B) or, human *PEX* cDNAs (Fig. 7C) were incubated in the presence of [D-Ala^2,Leu^5]enkephalin (500 μM) and hydrolysis products were resolved by HPLC as described in the *Experimental Procedures* section. Tyr-D-Ala-Gly was identified by chromatography of synthetic marker peptide.

While the *PEX* sequence preserves two of the residues critical for catalytic activity of NEP (equivalent to E^{646} and H^{711}), it lacks a residue equivalent to R^{102} shown to be crucial for the dipeptidylcarboxypeptidase activity of NEP. Therefore, unlike NEP, *PEX* has no dipeptidylcarboxypeptidase activity, but likely functions as an endopeptidase.

To examine recombinant human *PEX* for endopeptidase activity, cell membrane preparations from COS cells transiently expressing the protein were incubated with human PTH [1-38] or PTH [1-34] and the cleavage products were analyzed by reverse-phase high pressure liquid chromatography (HPLC), as shown in Fig. 8. Human PTH [1-38] was incubated with cell membrane preparations from vector transfected COS-7 cells (Fig. 8A) or from cells transiently expressing human *PEX* and hydrolysis products were resolved by HPLC (Fig. 8B). Chromatographic profile of products arising from the hydrolysis of PTH [1-34] when incubated with cell membranes from COS-7 cells transiently expressing *PEX* (Fig. 8C). The novel product with a molecular weight of

630 likely corresponds to the terminal pentapeptide DVHNF of human PTH [1-34].

A parallel preparation from vector transfected COS cells did not appreciably cleave PTH [1-38]. However, in the presence of *PEX*, both PTH peptides were hydrolyzed in a highly reproducible pattern resulting in the formation of several peaks that absorb at 214 nm. Mass spectrometry of the peptide materials recovered from two product peaks gave m/z values of 861 and 630, respectively. While the former product was present in hydrolysates from both PTH [1-38] and PTH [1-34], the latter product was identified only in the PTH [1-34] hydrolysate and likely corresponds to the carboxyl terminal pentapeptide DVHNF of human PTH [1-34]. These findings provide the first direct evidence that recombinant *PEX* possesses endopeptidase activity and suggest that its substrate specificity may not be restricted to the putative phosphatonin but may include other circulating hormones or perhaps bone-derived autocrine/paracrine regulatory factors that regulate renal phosphate handling.

DISCUSSION

To gain insight into the role of *PEX* in normal physiology we have cloned the human full-length cDNA and studied its expression, subcellular localization, and peptidase activity. The cloned human *PEX* cDNA encodes a protein whose deduced amino acid sequence is identical to the published partial (The HYP Consortium (1995) *Nature Genetics* **11**, 130-136) and to the full-length sequences reported more recently (Beck, L. et al. (1997) *J. Clin. Invest.* **99**, 1200-1209; Grieff, M. et al. (1997) *Biochem. Biophys. Res. Commun.* **231**, 635-639; Guo, R. and Quarles, L. D. (1997) *J. Bone Miner. Res.* **12**, 1009-1017). Its deduced topology is that of a type II integral membrane glycoprotein and in the pres-

ent study we have provided experimental evidence to support this prediction. We have shown that *PEX* is glycosylated in the presence of canine microsomal membranes and partitions exclusively in the detergent phase following extraction with Triton X-114, consistent with the prediction from sequence analysis that it is an integral membrane glycoprotein. Nevertheless, the observed hydrophobic nature of *PEX*, need not be attributed solely to it being an integral membrane protein. Lipophilic modification is known to cause cell membrane association, presumably through hydrophobic interaction of the modifying group with the lipid bilayer. Signaled by the C-terminal tetrapeptide CRLW motif, post-translational attachment of isoprenoids via a thioether linkage to the cysteine residue would be sufficient to promote effective membrane association. Further studies will be necessary to determine if such lipid modification of *PEX* does indeed take place. Of interest, however, is the observation that a nonsense mutation within this motif (R747Stop) has been reported to cosegregate with HYP and is likely to be associated with an inactive *PEX* gene product. Finally, the localization of *PEX* expressed in A293 cells is also consistent with the protein being membrane-associated and corroborates the sequence-based prediction that *PEX* is a type II integral membrane protein with its large C-terminal hydrophilic domain in the extracellular compartment. While protein expression was detected mostly on the cell surface, in some cells the signal was also localized intracellularly. This localization of the expressed protein would indicate that a portion of *PEX* activity is located in a membrane-bound compartment, possibly the Golgi membranes. The Golgi localization described for ECE-1 activity in cultured endothelial cells is proposed to promote the efficient conversion

of big endothelin-1 because of the co-localization and concentration of enzyme and substrate through the constitutive secretory pathway. It is possible then, that in parallel fashion, the *PEX* enzyme mediates both intracellular and cell-surface conversions of its putative substrate.

The finding that wild-type *PEX* transcripts are expressed in relative overabundance in OHO tumors poses a question in trying to understand the pathophysiology of these disorders. That is, how do we reconcile the apparently disparate observations that overexpression of *PEX* in OHO and loss of function in HYP patients, both lead to similar derangement in phosphate homeostasis? One of the physiological functions of *PEX* may well be the inactivation of a factor that normally promotes renal phosphate excretion (Fig. 9). The diagrams indicate events proposed to occur at the level of the proximal renal tubule. A putative circulating phosphaturic hormone (PHa) interacts with its renal receptor (PR) and inhibits phosphate reabsorption across the renal brush border membrane (-|) by decreasing NaPi activity. Downward arrows indicate the degree of phosphate excretion. *PEX* expressed predominantly in extrarenal tissues modulates the levels of circulating PHa by converting it to its inactive form (PHi).

In patients with OHO, the hyperphosphaturia that characterizes the syndrome would be the consequence of unregulated and excessive elaboration of the phosphaturic factor by the tumor. The modestly elevated *PEX* levels that we have documented in these tumors may arise either in response to the severe hypophosphatemia or to the abnormally high levels of the active phosphaturic factor. Yet, the increased *PEX* expression may not be sufficient to accommodate the increased substrate load, resulting in abnormally high circulating levels of the

active phosphaturic hormone. The inactivation of *PEX* observed in HYP patients would similarly cause decreased turnover of this humoral phosphaturic factor and thereby lead to renal phosphate wasting.

This model is also consistent with the observation that the *Hyp* phenotype is neither corrected nor transferred following cross transplantation of kidneys in normal and *Hyp* mice. Thus, when *Hyp* mice are engrafted with a normal kidney, phosphaturia ensues since circulating levels of the phosphaturic agent are excessive. On the other hand, engraftment of mutant kidneys in normal mice will not affect renal tubular phosphate handling of the recipients since circulating levels of the phosphaturic substance will be normally regulated by the enzymatic activity of extrarenal wild-type *PEX*. Indeed, analysis of the tissue distribution of *PEX* mRNA by RT-PCR has confirmed its expression in extrarenal tissues and particularly bone. Our present findings and those of others (Du, L. et al. (1996) *Genomics* **36**, 22-28; Beck, L. et al. (1997) *J. Clin. Invest.* **99**, 1200-1209; Grieff, M. et al. (1997) *Biochem. Biophys. Res. Commun.* **231**, 635-639; Guo, R. and Quarles, L. D. (1997) *J. Bone Miner. Res.* **12**, 1009-1017) showing high levels of *PEX* expression in cells of the osteoblast lineage would be consistent with the intrinsic osteoblast defect postulated to exist in HYP patients and in *Hyp* mice.

Finally, although the deduced structure of *PEX* clearly suggests that it is a metalloprotease, no peptidase activity had been ascribed to the protein. The preservation of the catalytic glutamate and histidine residues (equivalent to E^{646} and H^{711} of NEP; Fig. 2B) would argue for such an activity. In addition, the wide range of *PEX* mutations in HYP patients that align with regions required for protease activity in NEP suggests

that *PEX* also functions as a protease. Here, for the first time, we provide experimental evidence that recombinant *PEX* indeed functions as an endopeptidase. Unlike NEP, however, the protein does not possess dipeptidylcarboxypeptidase activity since it lacks a residue equivalent to R^{102} of NEP. Our unexpected observation that *PEX* effectively degrades PTH raises the question of whether circulating PTH is the putative phosphatonin. Although extracts from some OHO tumors have been reported to stimulate renal adenylate cyclase and this activity was inhibited by PTH antagonists, most studies have excluded PTH and PTH-related peptide (PTHrP) activity in OHO-associated tumors. Moreover, calcium homeostasis is generally preserved in patients with HYP. It is more likely, therefore, that the enzyme is rather promiscuous in its substrate specificity. *PEX* may indeed modulate PTH bioavailability and bioactivity, particularly at the level of the osteoblast, as well as the hormonal and paracrine/autocrine effects of factors produced by osteoblasts involved in regulating phosphate reabsorption and osteoblast maturation and mineralization. Although additional work will be required to clarify many of these issues, the availability of full-length human *PEX* cDNA now provides us with the opportunity to study the biology of *PEX*, identify its substrate(s), elucidate its role in pathological states characterized by dysregulated phosphate homeostasis, and determine its suitability as target for therapeutic intervention in the treatment of metabolic bone diseases.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following,

in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for the diagnosis of metabolic bone diseases in a patient, which comprises the step of determining the level of PTHrP in a biological sample of a patient wherein an alteration of PTHrP levels from that of a normal individual is indicative of metabolic bone diseases and/or metabolic bone diseases predisposition.

2. The method of claim 1, wherein said metabolic bone diseases is osteomalacia, osteoporosis, osteopetrosis or Paget's disease.

3. A method for the treatment of metabolic bone diseases, which comprises administering to a patient a compound for the modulation of *PEX* enzymatic activity modulates PTH and PTHrP levels that regulate osteoblast activity.

4. The method of claim 3, wherein said metabolic bone diseases is osteomalacia, osteoporosis, osteopetrosis or Paget's disease.

5. Use of a compound for the modulation of *PEX* enzymatic activity for the manufacture of a medicament for treating metabolic bone diseases, wherein said compound modulates PTH and PTHrP levels that regulate osteoblast activity.

6. The use of claim 5, wherein said metabolic bone diseases is osteomalacia, osteoporosis, osteopetrosis or Paget's disease.

7. A method for the treatment of metabolic bone diseases, which comprises modulating PTH and PTHrP levels that regulate osteoblast activity in a patient to modulate bone breakdown and bone formation.

8. The method of claim 8, wherein said metabolic bone diseases is osteomalacia, osteoporosis, osteopetrosis or Paget's disease.

9. Use of a compound for the modulation of PTH and PTHrP levels that regulate osteoblast activity for the treatment of metabolic bone diseases.

10. The use of claim 9, wherein said metabolic bone diseases is osteomalacia, osteoporosis, osteopetrosis or Paget's disease.

11. A non-human transgenic mammal to study the role of PEX in bone development and homeostasis, whose germ cells and somatic cells contain a PEX gene construct for expression of PEX in osteoblast consisting essentially of a recombinant PEX gene sequence under the control of a proximal promoter of a pro-a1(I) collagen gene, the PEX gene construct being introduced into the mammal, or an ancestor of the mammal, at an embryonic stage.

12. The non-human mammal of claim 11, which is a mouse and the proximal promoter is murine pro-a1(I) collagen gene.

13. The non-human mammal of claim 12, wherein said murine pro-a1(I) collagen gene is a 2.3 kb fragment thereof.

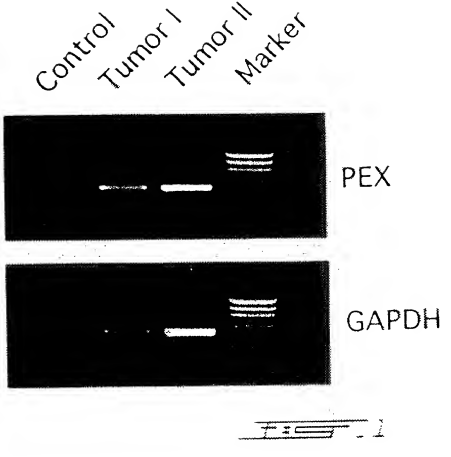

Fig. 1

09/806110

2/18

1 31
GAT CCA CTA GTA ACG GCC GCC AGT GTG GTG GAA TTC AAG GGA CTC ACA CAC TGA AAG AAT
61 91
ATC TTT GAT GAA GAC AAT TCA GGC AAG CAG AAT GAT TCT TGC AAC AGA ATT ACA TGA TTA
121 151
ATT GAG ATC TTG AAG TGG GTC CGG TGA ATC CTG GCC ACC TAA CTT ATC ATG ATT TGG GGG
181 211
AGT TTC ACG AGA ATC CAG TTT TGA TAA AAC AAT TGT TTT TTT CCT CCC CAA GTG ACT ATA
241 271
CAT TTA AAT AGC TAA AAC ATC TGT TCA GCA ACA TAG TAA AAC ATA TAT ACT CGG AAC GCT
301 331
TGA GAG AAG AGC CTG CCA AAC AGG GAC TTT GCT GAG GGA GAG CAC CAA GAT AAA GCA ACA
361 391
CTG TTT GTT TTG TCT AGT CAG GGG GGA AAG CCA AGG CAA CCA ATA TTT TGG TTT TTA TAA
421 451
TTT TCA TTT GTG AAG AAT TAT TTG AGA AAG GGT TGG CGA GGG GAG ATT TCC TGA CGG CAG
481 511
TTT CTT AAG CTG TCC ATT AGT AGA AGA GCA AGA GAG CCT TGG ATG TCA ACG CCT CGC TCT
541 571
TGA GAC CAG CCA CCA AAC CAC GAA AAG TGA CTT TCT TCT CGT GTG CTC TCT ACG GCC CTT
601 /1 631/10
CTG ATG GAA GCA GAA ACA GGG AGC AGC GTG GAG ACT GGA AAG AAG GCC AAC AGA GGC ACT
M E A E T G S S V E T G K K A N R G T
661/20 691/30
CGA ATT GCC CTG GTC GTG TTT GTC GGT GGC ACC CTA GTT CTG GGC ACG ATC CTC TTT CTA
R I A L V V F V G G T L V L G T I L F L

FIG. 2A (cont.)

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721/40                                      751/50
GTG AGT CAA GGT CTC TTA AGT CTC CAA GCT AAA CAG GAG TAC TGC CTG AAG CCA GAA TGC
 V   S   Q   G   L   L   S   L   Q   A   K   Q   E   Y   C   L   K   P   E   C
781/60                                      811/70
ATC GAA GCG GCT GCT GCC ATC TTA AGT AAA GTA AAT CTG TCT GTG GAT CCT TGT GAT AAT
 I   E   A   A   A   A   I   L   S   K   V  [N]  L   S   V   D   P   C   D   N
841/90                                      871/90
TTC TTC CGG TTC GCT TGT GAT GGC TGG ATA AGC AAT AAT CCA ATT CCC GAA GAT ATG CCA
 F   F   R   F   A   C   D   G   W   I   S   N   N   P   I   P   E   D   M   P
901/100                                     931/110
AGC TAT GGG GTT TAT CCT TGG CTG AGA CAT AAT GTT GAC CTC AAG TTG AAG GAA CTT TTG
 S   Y   G   V   Y   P   W   L   R   H   N   V   D   L   K   L   K   E   L   L
961/120                                     991/130
GAG AAA TCA ATC AGT AGA AGG CGG GAC ACC GAA GCC ATA CAG AAA GCC AAA ATC CTT TAT
 E   K   S   I   S   R   R   R   D   T   E   A   I   Q   K   A   K   I   L   Y
1021/140                                    1051/150
TCA TCC TGC ATG AAT GAG AAA GCG ATT GAA AAA GCA GAT GCC AAG CCA CTG CTA CAC ATC
 S   S   C   M   N   E   K   A   I   E   K   A   D   A   K   P   L   L   H   I
1081/160                                    1111/170
CTA CGG CAT TCA CCT TTC CGC TGG CCC GTG CTT GAA TCT AAT ATT GGC CCT GAA GGG GTT
 L   R   H   S   P   F   R   W   P   V   L   E   S   N   I   G   P   E   G   V
1141/180                                    1171/190
TGG TCA GAG AGA AAG TTC AGC CTT CTG CAG ACA CTT GCA ACG TTT CGT GGT CAA TAC AGC
 W   S   E   R   K   F   S   L   L   Q   T   L   A   T   F   R   G   Q   Y   S
1201/200                                    1231/210
AAT TCT GTG TTC ATC CGT TTG TAT GTG TCC CCT GAT GAC AAA GCA TCC AAT GAA CAT ATC
 N   S   V   F   I   R   L   Y   V   S   P   D   D   K   A   S   N   E   H   I
```

FIG. 2A (cont.)

```
1261/220                                                1291/230
TTG AAG CTG GAC CAA GCA ACA CTC TCC CTG GCC GTG AGG GAA GAC TAC CTT GAT AAC AGT
  L   K   L   D   Q   A   T   L   S   L   A   V   R   E   D   Y   L   D   N   S
1321/240                                                1351/250
ACA GAA GCC AAG TCT TAT CGG GAT GCC CTT TAC AAG TTC ATG GTG GAT ACT GCC GTG CTT
  T   E   A   K   S   Y   R   D   A   L   Y   K   F   M   V   D   T   A   V   L
1381/260                                                1411/270
TTA GGA GCT AAC AGT TCC AGA GCA GAG CAT GAC ATG AAG TCA GTG CTC AGA TTG GAA ATT
  L   G   A   N   S   S   R   A   E   H   D   M   K   S   V   L   R   L   E   I
1441/280                                                1471/290
AAG ATA GCT GAG ATA ATG ATT CCA CAT GAA AAC CGA ACC AGC GAG GCC ATG TAC AAC AAA
  K   I   A   E   I   M   I   P   H   E   N   R   T   S   E   A   M   Y   N   K
1501/300                                                1531/310
ATG AAC ATT TCT GAA CTG AGT GCT ATG ATT CCC CAG TTC GAC TGG CTG GGC TAC ATC AAG
  M   N   I   S   E   L   S   A   M   I   P   Q   F   D   W   L   G   Y   I   K
1561/320                                                1591/330
AAG GTC ATT GAC ACC AGA CTC TAC CCC CAT CTG AAA GAC ATC AGC CCC TCC GAG AAT GTG
  K   V   I   D   T   R   L   Y   P   H   L   K   D   I   S   P   S   E   N   V
1621/340                                                1651/350
GTG GTC CGC GTC CCG CAG TAC TTT AAA GAT TTG TTT AGG ATA TTA GGG TCT GAG AGA AAG
  V   V   R   V   P   Q   Y   F   K   D   L   F   R   I   L   G   S   E   R   K
1681/360                                                1711/370
AAG ACC ATT GCC AAC TAT TTG GTG TGG AGA ATG GTT TAT TCC AGA ATT CCA AAC CTT AGC
  K   T   I   A   N   Y   L   V   W   R   M   V   Y   S   R   I   P   N   L   S
1741/380                                                1771/390
AGG CGC TTT CAG TAT AGA TGG CTG GAA TTC TCA AGG GTA ATC CAG GGG ACC ACA ACT TTG
  R   R   F   Q   Y   R   W   L   E   F   S   R   V   I   Q   G   T   T   T   L
```

FIG - 2A (cont.)

```
1801/400                                                    1831/410
CTG CCT CAA TGG GAC AAA TGT GTA AAC TTT ATT GAA AGT GCC CTC CCT TAT GTT GGA
 L   P   Q   W   D   K   C   V   N   F   I   E   S   A   L   P   Y   V   G
1861/420                                                    1891/430
AAG ATG TTT GTA GAT GTG TAC TTC CAG GAA GAT AAG AAG GAA ATG ATG GAG GAA TTG GTT
 K   M   F   V   D   V   Y   F   Q   E   D   K   K   E   M   M   E   E   L   V
1921/440                                                    1951/450
GAG GGC GTT CGC TGG GCC TTT ATT GAC ATG CTA GAG AAA GAA AAT GAG TGG ATG GAT GCA
 E   G   V   R   W   A   F   I   D   M   L   E   K   E   N   E   W   M   D   A
1981/460                                                    2011/470
GGA ACG AAA AGG AAA GCC AAA GAA AAG GCG AGA GCT GTT TTG GCA AAA GTT GGC TAT CCA
 G   T   K   R   K   A   K   E   K   A   R   A   V   L   A   K   V   G   Y   P
2041/480                                                    2071/490
GAG TTT ATA ATG AAT GAT ACT CAT GTT AAT GAA GAC CTC AAA GCT ATC AAG TTT TCA GAA
 E   F   I   M   N   D   T   H   V   N   E   D   L   K   A   I   K   F   S   E
2101/500                                                    2131/510
GCC GAC TAC TTT GGC AAC GTC CTA CAA ACT CGC AAG TAT TTA GCA CAG TCT GAT TTC TTC
 A   D   Y   F   G   N   V   L   Q   T   R   K   Y   L   A   Q   S   D   F   F
2161/520                                                    2191/530
TGG CTA AGA AAA GCC GTT CCA AAA ACA GAG TGG TTT ACA AAT CCG ACG ACT GTC AAT GCC
 W   L   R   K   A   V   P   K   T   E   W   F   T   N   P   T   T   V   N   A
2221/540                                                    2251/550
TTC TAC AGT GCA TCC ACC AAC CAG ATC CGA TTT CCA GCA GGA GAG CTC CAG AAG CCT TTC
 F   Y   S   A   S   T   N   Q   I   R   F   P   A   G   E   L   Q   K   P   F
2281/560                                                    2311/570
TTT TGG GGA ACA GAA TAT CCT CGA TCT CTG AGT TAT GGT GCT ATA GGA GTA ATT GTC GGA
 F   W   G   T   E   Y   P   R   S   L   S   Y   G   A   I   G   V   I   V   G
```

FIG - 2A (cont.)

```
2341/580                                                2371/590
CAT GAA TTT ACA CAT GGA TTT GAT AAT AAT GGT AGA AAA TAT GAT AAA AAT GGA AAC CTG
 H   E   F   T   H   G   F   D   N   N   G   R   K   Y   D   K   N   G   N   L
2401/600                                                2431/610
GAT CCT TGG TGG TCT ACT GAA TCA GAA GAA AAG TTT AAG GAA AAA ACA AAA TGC ATG ATT
 D   P   W   W   S   T   E   S   E   E   K   F   K   E   K   T   K   C   M   I
2461/620                                                2491/630
AAC CAG TAT AGC AAC TAT TAT TGG AAG AAA GCT GGC TTA AAT GTC AAG GGG AAG AGG ACC
 N   Q   Y   S   N   Y   Y   W   K   K   A   G   L   N   V   K   G   K   R   T
2521/640                                                2551/650
CTG GGA GAA AAT ATT GCT GAT AAT GGA GGC CTG CGG GAA GCT TTT AGG GCT TAC AGG AAA
 L   G   E   N   I   A   D   N   G   G   L   R   E   A   F   R   A   Y   R   K
2581/660                                                2611/670
TGG ATA AAT GAC AGA AGG CAG GGA CTT GAG GAG CCT CTT CTA CCA GGC ATC ACA TTC ACC
 W   I   N   D   R   R   Q   G   L   E   E   P   L   L   P   G   I   T   F   T
2641/680                                                2671/690
AAC AAC CAG CTC TTC TTC CTG AGT TAT GCT CAT GTG AGG TGC AAT TCC TAC AGA CCA GAA
 N   N   Q   L   F   F   L   S   Y   A   H   V   R   C   N   S   Y   R   P   E
2701/700                                                2731/710
GCT GCC CGA GAA CAA GTC CAA ATT GGT GCT CAC AGT CCC CCT CAG TTT AGG GTC AAT GGT
 A   A   R   E   Q   V   Q   I   G   A   H   S   P   P   Q   F   R   V   N   G
2761/720                                                2791/730
GCA ATT AGT AAC TTT GAA GAA TTC CAG AAA GCT TTT AAC TGT CCA CCC AAT TCC ACG ATG
 A   I   S   N   F   E   E   F   Q   K   A   F   N   C   P   P   N   S   T   M
2821/740                *                               2851
AAC AGA GGC ATG GAC TCC TGC CGA CTC TGG TAG CTG GGA CGC TGG TTT ATG GCA TCC TGA
 N   R   G   M   D   S   C   R   L   W   @
```

FIG. 2A (cont.)

```
2881                                                2991
GAC AGT TGC ACA GTG CCA GCG GAG GCT GCA CTG AGC CTT CAT CGC CCA TTG CTT TAG GCC
2941                                                3031
TGG AGG AGC TTT CAT TTT TAG TGC ATT TTC ATT ATT TGG GTA GGT GAC CTG CTT GGA TCT
3001                                                3091
AGA CAG CAT CTG TTC AAA GTT GTA GGG CTT ATA AAG TGG AAT ATA AGA AGA ACT AAG TAT
3051                                                3121
GTT TCT TTA GAA AAT CAA ACC AAC AAA AAT AAA TCC CTA GGC TAC TTT TGT TAA AAA AAA
AAA AAA AAA A
```

FIG - 2A (cont.)

```
          10        20        30        40        50        60
hPEX  EAETGSSVETGKKANRGTRIALVVFVGGTLVLGTILFLVSQGLLSLQAKQEYCLKPECIE
      :....... .   :...  : .....  . :::   ... :.   :.      . :  ..::.
hNEP  ESQMDITDINTPKPKKKQRWTPLEISLSVLVLLLTIIAVTMIALYATYDDGICKSSDCIK
          10        20        30        40        50        60

             70        80        90       100       110       120
hPEX  AAAAILSKVNLSVDPCDNFFRFACDGWISNNPIPEDMPSYGVYPWLRHNVDLKLKELLEK
      .:: .. ... . .:: .::..::.::.    : :::   ..:: .   ::. ... ::..:..
hNEP  SAARLIQNMDATTEPCTDFFKYACGGWLKRNVIPETSSRYGNFDILRDELEVVLKDVLQE
          70        80        90       100       110       120

            130       140       150       160       170       180
hPEX  SISRRRDTEAIQKAKILYSSCMNEKAIEKADAKPLLHILRHSPFRWPVLESNIGPEGVWS
      ...    :..:.:::: ::.::.:: ::.   ...::: .:... ..:::    : ... . :
hNEP  PKT--EDIVAVQKAKALYRSCINESAIDSRGGEPLLKLLPDI-YGWPVATENWEQKYGAS
           130       140       150       160        170       180

            190       200       210       220       230       240
hPEX  ERKFSLLQTLATFRGQYSNSVFIRLYVSPDDKASNEHILKLDQATLSLAVREDYLDNSTE
        .  ...: . ..:.. :.: :.:..::: :   :.. .::..:.:. :..:   ...
hNEP  W---TAEKAIAQLNSKYGKKVLINLFVGTDDKNSVNHVIHIDQPRLGLPSRDYYECTGIY
             190       200       210       220       230
```

FIG. 2B (cont.)

```
                250       260       270       280       290
hPEX    AKSYRDALY-KFMVDTAVLLGANSSRAEH---DMKSVLRLEIKIAEIMIPHENRT-SEA
        ..... .. .:.. ..... .....:.   .:. :. ::....::   .  .:.:. .
hNEP    KEACTAYVDFMISVARLIRQEERLPIDENQLALEMNKVMELEKEIANATAKPEDRNDPML
        240       250       260       270       280       290

                300       310       320       330       340       350
hPEX    MYNKMNISEL-SAMIPQFDWLGYIK-KVIDTRLYPHLKDISPSENVVVRVPQYFKDLFRI
        .::::..... ..... ..... ..... :.  :.:::. .:.:.. . : .:
hNEP    LYNKMTLAQIQNNFSLEINGKPFSWLNFTNEIMSTVNISITNEEDVVVYAPEYLTKLKPI
        300       310       320       330       340       350

                360       370       380       390       400       410
hPEX    LGSERKKTIANYLVWRMVVSRIPNLSRRFQYRWLEFSRVIQGTTTLLPQWDKCVNFIESA
        :  . . . : :: .  .....:::.... .. .:..... :::.  . : .:.:. .
hNEP    LTKYSARDLQNLMSWRFIMDLVSSLSRTYKESRNAFRKALYGTTSETATWRRCANYVNGN
        360       370       380       390       400       410
```

FIG - 2B (cont.)

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```
             420       430       440       450       460       470
hPEX    LPYVVGKMFVDVYFQEDKKEMMEELVEGVRWAFIDMLEKENEWMDAGTKRKAKEKARAVL
        . ..:...:.. : .. : ..:.:.. .: .:: .:. .  ::::.::..:.::::.:.
hNEP    MENAVGRLYVEAAFAGESKHVVEDLIAQIREVFIQTLD-DLTWMDAETKKRAEEKALAIK
       420       430       440       450        460       470

             480       490       500       510       520       530
hPEX    AKVGYPEFIM-NDTHVNEDLKAIKFSEADYFGNVLQTRKYLAQSDFFWLRKAVPKTEWFT
        ...:::. :. ::. .: .  .... :..::.:..:..:..     .  ::. : : ::..
hNEP    ERIGYPDDIVSNDNKLNNEYLELNYKEDEYFENIIQNLKFSQSKQLKKLREKVDKDEWIS
        480       490       500       510       520       530

              540       550       560       570       580       590
hPEX    NPTTVNAFYSASTNQIRFPAGELQKPFFWGTEYPRSLSYGAIGVIVGHEFTHGFDNNGRK
         .. :::::...::: :::: :: :::.. ..  ::.::.::..:::.::::::.:::.
hNEP    GAAVVNAFYSSGRNQIVFPAGILQPPFFSAQQSN-SLNYGGIGMVIGHEITHGFDDNGRN
        540       550       560       570        580       590
```

FIG. 2B (cont.)

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```
               600       610       620       630        640       650
hPEX      YDKNGNLDPWWSTESEEKFKEKTKCMINQYSNYYWKKAG-LNVKGKRTLGENIADNGGLR
          ..:.:.:. ::. .:. .:::...::..::.:..:  :: ....:. ::::::::::::.
hNEP      FNKDGDLVDWWTQQSASNFKEQSQCMVYQYGNFSWDLAGGQHLNGINTLGENIADNGGLG
           600       610       620       630       640       650

                660       670       680       690       700       710
hPEX      EAFRAYRKWINDRRQGLEEPLLPGITFTNNQLFFLSYAHVRCNSYRPEAAREQVQIGAHS
          .:.:::.   :....  ::  ::::. ....:::::..:.:.: .:::: :   .....::
hNEP      QAYRAYQ---NYIKKNGEEKLLPGLDLNHKQLFFLNFAQVWCGTYRPEYAVNSIKTDVHS
           660          670       680       690       700       710

                720       730       740
hPEX      PPQFRVNGAISNFEEFQKAFNCPPNSTMNRGMDSCRLW
          :  ::..:.. : .:: .::.:. :: ::...  ::.:
hNEP      PGNFRIIGTLQNSAEFSEAFHCRKNSYMNPEKK-CRVW
              720       730       740        750
```

FIG. 2B (cont.)

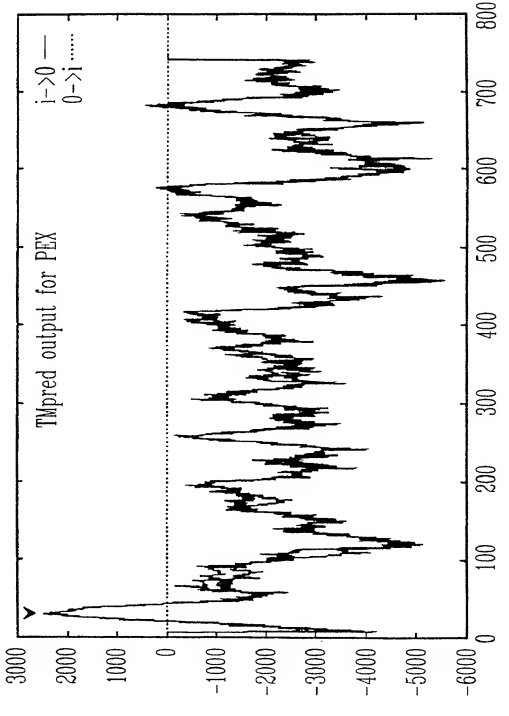
TMpred output for PEX
i->O ——
O->i .....
3000
2000
1000
0
-1000
-2000
-3000
-4000
-5000
-6000
0
100
200
300
400
500
600
700
800
Amino acid

Fig - 2C

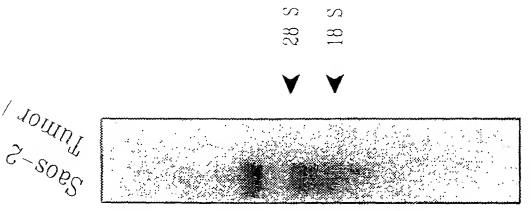

Fig - 4

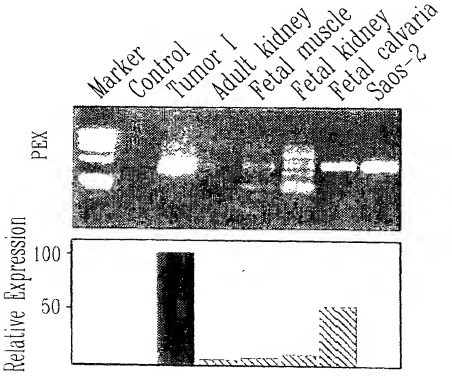

Fig - 3

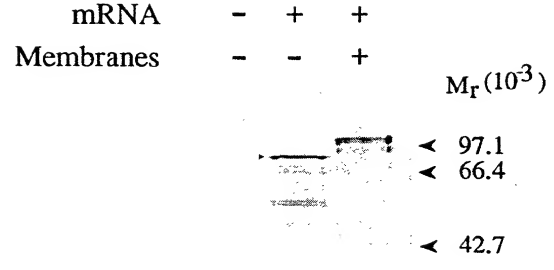

Fig. 5

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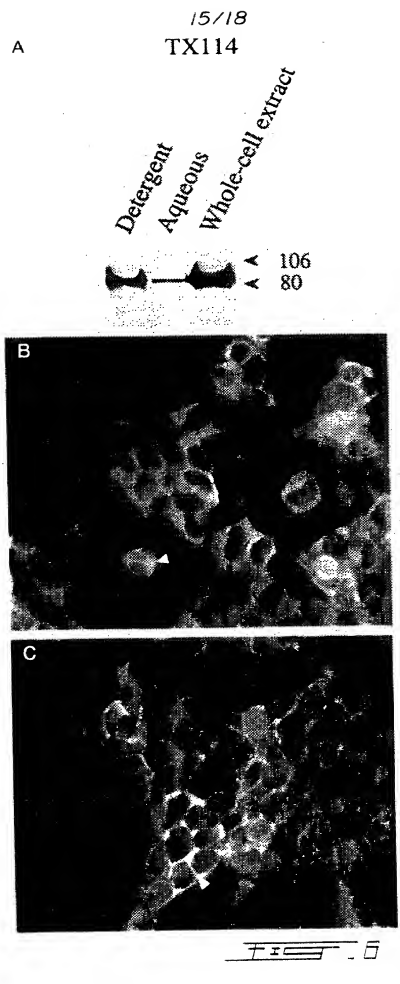

Fig. 6

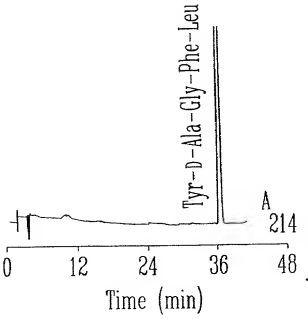

FIG. 7A

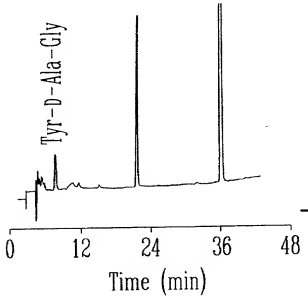

FIG. 7B

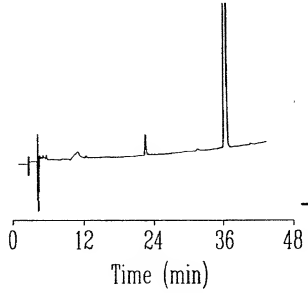

FIG. 7C

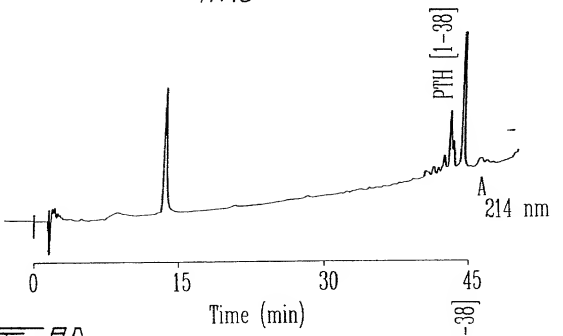

Fig. 8A

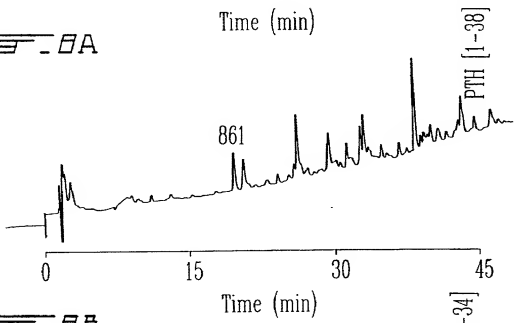

Fig. 8B

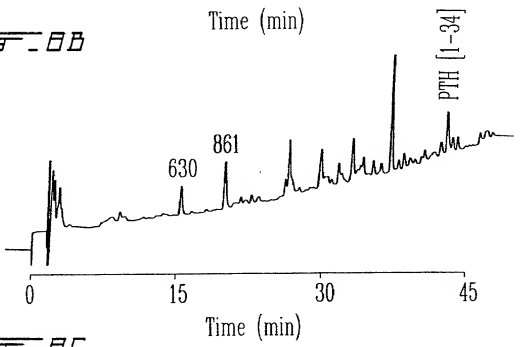

Fig. 8C

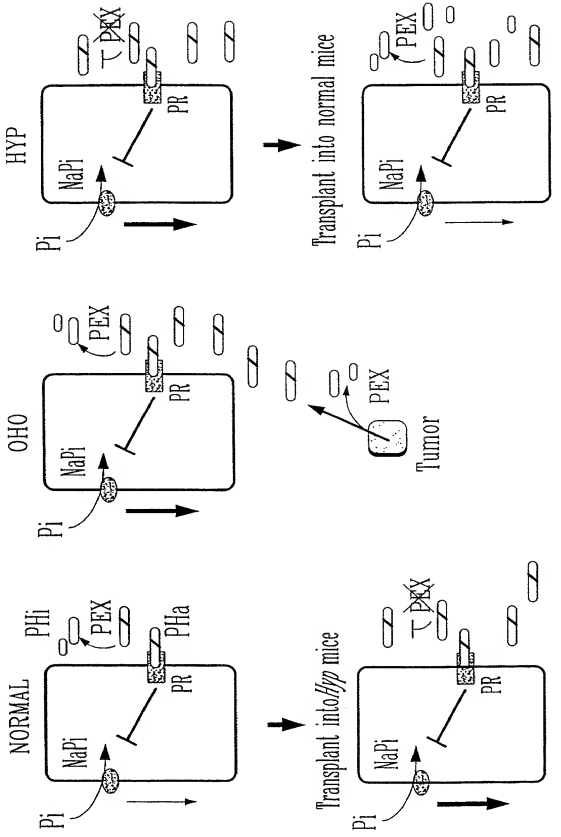
NORMAL
PHi
PEX
PHa
NaPi
PR
Pi
Transplant into hyp mice
HYP
PEX
NaPi
PR
Pi
Transplant into normal mice
OHO
PEX
NaPi
PR
Pi
PEX
Tumor

Fig - 9.

DECLARATION AND POWER OF ATTORNEY
(Attorney Docket No. 109.647.121)

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship is as stated below next to my name.

I believe that I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

the specification of which (check only one):

[ ] is attached hereto.

[X] was filed as United States Patent Application
Serial No. 09/805,110
on _____
and was amended
on _____
(if applicable)

[ ] was filed as PCT Patent Application
Serial No. _____
on _____
and was amended under PCT Article 19
on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of the claims of this application in accordance with Title 37, Code of Federal Regulations, Sections 1.56(a) and 1.56(b).

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. §119(a)-(d) or 365(b):

COUNTRY (if PCT indicate PCT)	APPLICATION NUMBER	DATE OF FILING	PRIORITY CLAIMED UNDER 35 U.S.C. §119(a)-(b) or 365(b) (YES/NO)
PCT	CA99/00895	September 27, 1999	Yes
Canada	2,245,903	September 28, 1998	Yes

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional patent application(s) listed below:

APPLICATION NUMBER	DATE OF FILING	STATUS: (PENDING OR ABANDONED)

I hereby claim the benefit under Title 35, United States Code, § 120 or 365(c) of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112. I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56 which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATION OR PCT INTERNATIONAL APPLICATION(S) DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. § 120 or 365(c):

APPLICATION NUMBER	DATE OF FILING (day, month, year)	STATUS: (PATENTED, PENDING OR ABANDONED)

POWER OF ATTORNEY: As named inventors, we hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith

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Sally Byrne	40,545	Rajesh Vallabh	35,761
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Nels Lippert	25,888	Dominic Massa	44,905
Gregory S. Discher	42,488	Irah H. Donner	35,120

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David Cerveney	44,600	David Cavanaugh	36,476
Irah H. Donner	35,120	Gregory S. Discher	42,488
Anthony Kahng	42,704	Maria Maebius	42,967
Cynthia Nicholson	36,880	Tamara Pertmer	P47,856
MaryRose Scozzafava	36,268	Victor Souto	33,458
Leonid Thenor	39,397	Michael J. Twomey	38,349
Gary A. Walpert	26,098	Lisa Wilson	34,045
Joseph Haag	42,612	Wendy A. Haller	35,177

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Wherefore I petition that letters patent be granted to me for the invention or discovery described and claimed in the attached specification and claims, and hereby subscribe my name to said specification and claims and to the foregoing declaration, power of attorney, and this petition.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements

were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Inventor's signature _____ Date 20/8/01

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(4) over

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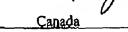
3 - 00 **Full name of additional joint inventor:** Mark L. Lipman

Inventor's signature  Date Aug 29, 2001

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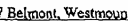
4 - 00 **Full name of additional joint inventor:** Janet E. Henderson

Inventor's signature  Date August 30, 2001

Country of Citizenship: Canada

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KARAPLIS, Andrew C.
GOLTZMAN, David
LIPMAN, Mark L.
HENDERSON, Janet E.

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Tyr Arg Lys Trp Ile Asn Asp Arg Arg Gln Gly Leu Glu Glu Pro Leu
            660                 665                 670
Leu Pro Gly Ile Thr Phe Thr Asn Asn Gln Leu Phe Phe Leu Ser Tyr
        675                 680                 685
Ala His Val Arg Cys Asn Ser Tyr Arg Pro Glu Ala Ala Arg Glu Gln
    690                 695                 700
Val Gln Ile Gly Ala His Ser Pro Pro Gln Phe Arg Val Asn Gly Ala
705                 710                 715                 720
Ile Ser Asn Phe Glu Glu Phe Gln Lys Ala Phe Asn Cys Pro Pro Asn
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Ser Thr Met Asn Arg Gly Met Asp Ser Cys Arg Leu Trp
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Lys Lys Gln Arg Trp Thr Pro Leu Glu Ile Ser Leu Ser Val Leu Val
            20                  25                  30
Leu Leu Leu Thr Ile Ile Ala Val Thr Met Ile Ala Leu Tyr Ala Thr
        35                  40                  45
Tyr Asp Asp Gly Ile Cys Lys Ser Ser Asp Cys Ile Lys Ser Ala Ala
    50                  55                  60
Arg Leu Ile Gln Asn Met Asp Ala Thr Thr Glu Pro Cys Thr Asp Phe
65                  70                  75                  80
Phe Lys Tyr Ala Cys Gly Gly Trp Leu Lys Arg Asn Val Ile Pro Glu
                85                  90                  95
Thr Ser Ser Arg Tyr Gly Asn Phe Asp Ile Leu Arg Asp Glu Leu Glu
            100                 105                 110
Val Val Leu Lys Asp Val Leu Gln Glu Pro Lys Thr Glu Asp Ile Val
        115                 120                 125
Ala Val Gln Lys Ala Lys Ala Leu Tyr Arg Ser Cys Ile Asn Glu Ser
    130                 135                 140
Ala Ile Asp Ser Arg Gly Gly Glu Pro Leu Leu Lys Leu Leu Pro Asp
145                 150                 155                 160
Ile Tyr Gly Trp Pro Val Ala Thr Glu Asn Trp Glu Gln Lys Tyr Gly
                165                 170                 175
Ala Ser Trp Thr Ala Glu Lys Ala Ile Ala Gln Leu Asn Ser Lys Tyr
            180                 185                 190
Gly Lys Lys Val Leu Ile Asn Leu Phe Val Gly Thr Asp Asp Lys Asn
        195                 200                 205
Ser Val Asn His Val Ile His Ile Asp Gln Pro Arg Leu Gly Leu Pro
    210                 215                 220
Ser Arg Asp Tyr Tyr Glu Cys Thr Gly Ile Tyr Lys Glu Ala Cys Thr
225                 230                 235                 240
Ala Tyr Val Asp Phe Met Ile Ser Val Ala Arg Leu Ile Arg Gln Glu
                245                 250                 255
Glu Arg Leu Pro Ile Asp Glu Asn Gln Leu Ala Leu Glu Met Asn Lys
            260                 265                 270
Val Met Glu Leu Glu Lys Glu Ile Ala Asn Ala Thr Ala Lys Pro Glu
        275                 280                 285
Asp Arg Asn Asp Pro Met Leu Leu Tyr Asn Lys Met Thr Leu Ala Gln
    290                 295                 300
Ile Gln Asn Asn Phe Ser Leu Glu Ile Asn Gly Lys Pro Phe Ser Trp
305                 310                 315                 320
Leu Asn Phe Thr Asn Glu Ile Met Ser Thr Val Asn Ile Ser Ile Thr
                325                 330                 335
Asn Glu Glu Asp Val Val Val Tyr Ala Pro Glu Tyr Leu Thr Lys Leu
            340                 345                 350
Lys Pro Ile Leu Thr Lys Tyr Ser Ala Arg Asp Leu Gln Asn Leu Met
        355                 360                 365
Ser Trp Arg Phe Ile Met Asp Leu Val Ser Ser Leu Ser Arg Thr Tyr
    370                 375                 380
Lys Glu Ser Arg Asn Ala Phe Arg Lys Ala Leu Tyr Gly Thr Thr Ser
385                 390                 395                 400
Glu Thr Ala Thr Trp Arg Arg Cys Ala Asn Tyr Val Asn Gly Asn Met
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                405                 410                 415

Glu Asn Ala Val Gly Arg Leu Tyr Val Glu Ala Ala Phe Ala Gly Glu
            420                 425                 430
Ser Lys His Val Val Glu Asp Leu Ile Ala Gln Ile Arg Glu Val Phe
        435                 440                 445
Ile Gln Thr Leu Asp Asp Leu Thr Trp Met Asp Ala Glu Thr Lys Lys
    450                 455                 460
Arg Ala Glu Glu Lys Ala Leu Ala Ile Lys Glu Arg Ile Gly Tyr Pro
465                 470                 475                 480
Asp Asp Ile Val Ser Asn Asp Asn Lys Leu Asn Asn Glu Tyr Leu Glu
                485                 490                 495
Leu Asn Tyr Lys Glu Asp Glu Tyr Phe Glu Asn Ile Ile Gln Asn Leu
            500                 505                 510
Lys Phe Ser Gln Ser Lys Gln Leu Lys Lys Leu Arg Glu Lys Val Asp
        515                 520                 525
Lys Asp Glu Trp Ile Ser Gly Ala Ala Val Val Asn Ala Phe Tyr Ser
    530                 535                 540
Ser Gly Arg Asn Gln Ile Val Phe Pro Ala Gly Ile Leu Gln Pro Pro
545                 550                 555                 560
Phe Phe Ser Ala Gln Gln Ser Asn Ser Leu Asn Tyr Gly Gly Ile Gly
                565                 570                 575
Met Val Ile Gly His Glu Ile Thr His Gly Phe Asp Asp Asn Gly Arg
            580                 585                 590
Asn Phe Asn Lys Asp Gly Asp Leu Val Asp Trp Trp Thr Gln Gln Ser
        595                 600                 605
Ala Ser Asn Phe Lys Glu Gln Ser Gln Cys Met Val Tyr Gln Tyr Gly
    610                 615                 620
Asn Phe Ser Trp Asp Leu Ala Gly Gly Gln His Leu Asn Gly Ile Asn
625                 630                 635                 640
Thr Leu Gly Glu Asn Ile Ala Asp Asn Gly Gly Leu Gly Gln Ala Tyr
                645                 650                 655
Arg Ala Tyr Gln Asn Tyr Ile Lys Lys Asn Gly Glu Glu Lys Leu Leu
            660                 665                 670
Pro Gly Leu Asp Leu Asn His Lys Gln Leu Phe Phe Leu Asn Phe Ala
        675                 680                 685
Gln Val Trp Cys Gly Thr Tyr Arg Pro Glu Tyr Ala Val Asn Ser Ile
    690                 695                 700
Lys Thr Asp Val His Ser Pro Gly Asn Phe Arg Ile Ile Gly Thr Leu
705                 710                 715                 720
Gln Asn Ser Ala Glu Phe Ser Glu Ala Phe His Cys Arg Lys Asn Ser
                725                 730                 735
Tyr Met Asn Pro Glu Lys Lys Cys Arg Val Trp
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